The Role of Serotonergic (5-HT) Neuromodulation

in Respiratory Chemosensitivity

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The Role of Serotonergic (5-HT) Neuromodulation in Respiratory Chemosensitivity

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Abstract

Breathing must be regulated to maintain appropriate levels of oxygen and carbon dioxide. Breathing may be influenced by serotonergic (5-HT) neurons, sensitive to CO_2, which activate the brain's respiratory network. However, this role of 5-HT neurons as CO_2-sensitive chemoreceptors in unanesthetized animals is unclear. This study used an unanesthetized *in situ* perfused rat brainstem preparation to test the hypothesis that 5-HT neurons contribute to CO_2 ventilatory responses. Changes in phrenic nerve discharge patterns were monitored as gas-saturated solutions supplying the preparation were switched from 5%CO_2 to 7%CO_2 (balance O_2). The importance of 5-HT neurons was identified by comparing responses before and after application of the specific serotonin 1A receptor agonist 8-hydroxy-(dipropylamino) tetralin hydrobromide (8-OH-DPAT; at doses of 0, 1.5, 3, and 6 uM in the perfusate). The action of 8-OH-DPAT is to inhibit 5-HT neurons and reduce synaptic 5-HT release. Results indicated that changes in phrenic burst pattern similar to hypercapnic ventilatory responses observed *in vivo*, were greatly disrupted by 8-OH-DPAT treatment. These results illustrate that activation of 5-HT neurons is critical for CO_2 chemosensitivity in this *in situ* preparation and suggest that these neurons may play a key role in the regulation of breathing in otherwise intact animals.

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1.0 Neural Control of Breathing

Respiration is a vital, complex process and must be regulated with precision. It facilitates oxygenation of the body's tissues, elimination of CO_2, and the associated acid-base balance that is necessary for homeostasis in aerobic organisms (von Euler 1986). Chemical homeostasis involves a delicate balance between CO_2, O_2 and pH levels. When respiration is inadequate, CO_2 accumulates. Extreme excesses of CO_2 molecules result in dangerously acidic pH levels ($H_2O + CO_2 \leftrightarrow H_2CO_3 \leftrightarrow H_+ + HCO_3^-$), and at low pH levels (high [H+]) the respiratory center in the medulla becomes stimulated to change ventilation appropriately (Remmers 1999). It is important to keep CO_2 levels within a very narrow range, as even small changes in the concentration of protons in the blood (less than 0.1 uM) can be fatal (Richerson 2004). In order to maintain pH within physiological limits there are neurons in the brainstem that monitor the levels of CO_2 via changes in pH. These neurons transfer information about CO_2 to the respiratory network, where breathing is adjusted accordingly (Feldman *et al.* 2003, Richerson 2004).

The neural network that controls ventilation (the respiratory network) has a hierarchical organization with voluntary and involuntary components, including respiratory sensors, controllers and effectors. Regulation of CO_2, O_2 and pH is accomplished by the respiratory musculature whose activities are controlled by the central nervous system (Feldman and McCrimmon 1999). This activity is further modulated by peripheral input from various sensory neurons which are important in maintaining chemical homeostasis, such as the pulmonary stretch receptors in the lungs (Harris 2003) and chemoreceptors in the carotid bodies (Day 2005). Changes in

breathing are primarily autonomic: the respiratory network monitors CO_2 and O_2 and responds appropriately.

Ventilation of the lungs is central to respiration, as eliminating CO_2 from the blood helps increase the pH of blood. Intercostal and diaphragm muscles actively contract to pull O_2-laden air into the lungs, and gas exchange occurs when inhaled O_2 passes into tiny alveoli in the lungs and diffuses through pulmonary capillaries into arterial blood. CO_2 diffuses in the opposite direction, from capillary blood to the alveoli, reducing blood pH The diaphragm relaxes on exhalation, in conjunction with elastic recoil of the chest and, in some cases, contraction of abdominal muscles, and CO_2-laden air is forced out of the lungs. It is a perpetual, dynamic, rhythmic process that normally proceeds smoothly and subconsciously.

Although rhythmic and continual, respiration is not always steady; it exhibits considerable plasticity, which matches breathing to metabolic demands (Mitchell and Johnson 2000). In the face of long term physiological changes like development, aging, pregnancy, changes in weight, and brain injury, the brain can make precise adjustments to preserve adequate respiration. Respiration also changes almost immediately with changes that increase or decrease O_2 consumption: exercise, posture, sleep state, massive blood loss, and intensely emotional situations result in changes in the breathing pattern (Kestin 1992). Respiration remains constantly responsive to challenges affecting O_2, pH, and CO_2 (Feldman *et al* 2003). Although mainly under subconscious control, respiration can also be changed voluntary. It is an incessant and precise cycle.

1.1 Respiratory pattern generation

Respiration normally occurs in an automatic, rhythmic cycle . The respiratory network is active from birth until death, without significant pause. The involuntary control center of respiration is located in the brainstem, particularly in the medulla and pons. This rhythmically active network regulates the cycle of inspiration and expiration. The network is driven by central chemoreceptors and other sources of tonic input, like stretch receptors in the lungs and peripheral chemoreceptors (Feldman *et al.* 2003). As it attempts to maintain an appropriate balance of O_2 and CO_2, the respiratory network serves as a kind of metronome, providing directions to motor neurons innervating the respiratory muscles, resulting in muscle movements necessary for inspiration and expiration. Although the respiratory rhythm can be generated in the absence of afferent fibers (von Euler 1986), peripheral input helps to ensure that the rhythm is physiologically appropriate. Exactly how the network is organized and what mechanisms underlie rhythmogenesis are topics of debate.

The respiratory network must monitor dynamic levels of O_2 and CO_2 (via related changes in pH) and adjust breathing appropriately (von Euler 1986). Since the respiratory centers in the medulla and pons have no, or inadequate, sensitivity to pH on their own (Nattie and Li 2001; Feldman and Del Negro 2003), they must use chemosensitive neurons of the respiratory network to sense changes in pH (Nattie 1999). Afferent inputs provide chemical information to the network, which integrates the information and produces appropriate respiratory outputs that match ventilation to

metabolic demands (McCrimmon *et al.* 1995). It is vital to define which neurotransmitters may be responsible for modulating the respiratory network.

Two major areas of the brainstem have been identified which may contribute to the rhythm of respiration: the pre-inspiratory area (preI) in the retrotrapezoid nucleus/ parafacial respiratory group and the pre-Botzinger complex (Feldman and Del Negro 2006). The preI area contains neurons known to fire immediately before and after the inspiratory burst, and they retain rhythmic activity in the presence of synaptic blockade and in the absence of high potassium (Onimaru *et al.*1990). The preBotzinger nucleus is proposed as the site where respiratory rhythm is generated (Smith *et al.* 1991), and preBotz neurons likely work in concert with preI neurons to produce the respiratory rhythm in intact mammals (see Feldman and Del Negro 2006, Wilson *et al.* 2006, for review). Although it is widely accepted that rhythm generators like the preI and the preBotzinger exist in the respiratory network, details of their locations and interactions are unclear and were not an integral part of this study. However, it was relevant to consider what inputs, if any, may ultimately be affecting the rhythm-generating neurons. Wang *et al.* (2002) found that lesioning neurons in the respiratory complex results in inappropriate responses to CO_2. Although the respiratory network produces a regular breathing rhythm, tonic inputs may also be required to maintain adequate respiration. Sources of tonic drive include chemoreceptors, state dependence/higher brain centers, and O_2- sensitive inputs from carotid bodies. In addition to these, breathing can be controlled voluntarily by the forebrain (McCrimmon 1995). This fusion of inputs results in a process with a variety of influences, difficult to tease out experimentally.

Experiments using isolated or transected brainstems, while valuable in isolating rhythmic neurons, may remove natural tonic inputs (Wilson *et al.* 2006). Experiments using anesthetized animals retain the tonic network but respiration may be affected by anesthesia (Kestin 1992). It seems, therefore, that an extremely useful experimental model would be the intact, unanesthetized brainstem. The perfused rat brainstem used for this study includes the respiratory network, including sources of tonic drive, and exhibits respiratory patterns and ventilatory reflexes resembling those of intact animals (Paton 1996, St. John and Paton 2000).

1.2 Neuromodulation and chemosensitivity

Neuromodulation allows the nervous system to adapt its control of physiological functions to a changing environment. Neuromodulation is the process by which neurotransmitters or other substances are released into broad regions of the nervous system and have an effect on entire populations of neurons downstream. This is more efficient, but less precise, than a typical synaptic connection where one presynaptic neuron communicates with one postsynaptic neuron. For example, neurons near the bloodstream can sense changes in arterial blood pH and immediately convey this information to hundreds or thousands of efferent neurons in the respiratory network that can influence ventilation (Richerson 2004). Neuropeptides, biogenic amines, and neurotransmitters like acetylcholine, dopamine, serotonin and histamine are all capable of modulation (Katz and Frost, 1996). Serotonin (5-hydroxytryptamine, 5-HT) may play a role in the modulation of breathing (Bradley *et al.* 2002, Feldman *et al.* 2003, Onimaru *et*

al. 1998, Pena and Ramirez 2002). For example, application of specific serotonin receptor ligands exerts an excitatory effect on respiratory activity both *in vitro* (Onimaru *et al.*1998) and *in vivo* (Lalley *et al.* 1994, Cayetanot *et al.* 2002). This study focuses on serotonergic neurons (neurons which secrete serotonin) and their role as neuromodulators of the respiratory network.

Neurons that can detect CO_2 or pH are classified as chemoreceptors. Respiratory chemoreceptors are cells that possess an intrinsic sensitivity to pH and/or CO_2, as well as anatomical connections in the central nervous system that enable them to modulate the respiratory network (Guyenet *et al.* 2005). In this manner, chemoreceptors in the respiratory network provide the chemical drive to change breathing in response to changing O_2 and CO_2 levels. There are two types of chemoreceptors: "central" chemoreceptors are located in the brainstem and respond to arterial P_{CO2} via extracellular pH (Eldridge 1984) or intracellular pH, and "peripheral" chemoreceptors in the carotid bodies that respond to arterial P_{CO2} in a P_{O2}- and glucose-dependent manner (Feldman *et al.* 2003, Richerson, 2004; Putnam *et al.* 2004). The network of rhythmic respiratory neurons relies mainly on central chemoreceptors, which may have excitatory or inhibitory effects, along with other sources of excitatory drive (Feldman *et al.* 2003). The way in which chemosensitive neurons integrate at the cellular and network levels is uncertain (Feldman *et al.* 2003), and the details of how they modulate respiration are also under investigation (Richerson 2004, Guyenet *et al.* 2005).

Recently, great progress has been made towards identifying specific neuronal types which may be central chemoreceptors. These include serotonergic neurons of the

midline raphe (Richerson 2004), serotonergic neurons in the medulla with adjacent neurons that express neurokinin-1 (Nattie *et al.* 2004), glutamatergic neurons of the retrotrapezoid nucleus (Mulkey *et al.* 2004), and noradrenergic neurons of the locus coeruleus (Putnam *et al.* 2004). There are also other neurons without known neurotransmitter content that may act as central chemoreceptors; for example, neurons in the nucleus tractus solitarius, preBotzinger complex, hypothalamus and cerebellum (Richerson 1998; Feldman *et al.* 2003, Putnam *et al.* 2004). Although the existence of central pH/CO_2 chemoreception is widely recognized, neither the neuron type (serotonergic, glutamatergic, noradrenergic, etc.) nor the precise location of the central chemoreceptors has been definitively established (for review, see Guyenet 2005 and Richerson 2005). The evidence, however, supports the role of serotonergic neurons of the midbrain in central respiratory chemoreception.

1.3 Serotonergic neurons and chemosensitivity

Serotonin (5-HT) is a monoaminergic neurotransmitter that is made in neuronal cell bodies in the central nervous system and in enterochromaffin cells in the gastrointestinal tract (Richter 2003). In the brain, it has enormous influence over a variety of functions, including motor activity, cognition, mood, arousal, breathing, and cardiovascular function. Serotonin is synthesized from the amino acid L-tryptophan for release into the synapse, where it binds to 5-HT receptors on the postsynaptic neuron and presynaptic cell body (Richter *et al.* 2003). At least 15 5-HT receptor subtypes have been identified, and the majority of these act as slow, G-protein-linked receptors, through

second messenger systems and adenylate cyclase (Barnes and Sharp, 1999). Specific serotonin receptor subtypes have been implicated in various disorders, including schizophrenia, depression, anxiety, obsessive compulsive disorder, and migraine. The physiological function of each receptor subtype has not been established and is still under investigation. (The 5-HT_{1A} receptor family in the raphe nuclei has been implicated in respiration, and is discussed in further detail in Section 1.4). Serotonergic activity in the synapse is terminated when serotonin molecules are recycled by the serotonin transporter (SERT) or degraded into metabolic byproducts by monoamine oxidases (Pauwels 1997).

Serotonergic neurons in the medulla may have a role as central respiratory chemoreceptors (Wang *et al.* 2002, Bradley *et al.* 2002), because of their chemosensitivity and occurrence in regions known to influence breathing (Richerson 2004). According to Richerson (1998), Putnam *et al.* (2004), and Guyenet *et al.* (2005), pH and/or CO_2 chemoreceptor candidates must meet two criteria: they must possess intrinsic chemosensitivity to physiologically relevant changes in pH/CO_2, and they must have the capability to appropriately affect respiratory output. Serotonergic neurons meet both criteria (Richerson 2004).

1.3.1 Chemosensitivity of serotonergic neurons

Acidification of serotonergic neurons in brain tissue slices, neuron culture, and *in vivo* preparations induces changes in their firing rates, and reveals their intrinsic chemosensitivity, as demonstrated in the following examples. Wang *et al.* (1998) showed that cells of the rat medullary raphe are stimulated by an increase in CO_2,

increasing their firing rate threefold in response to an increase P_{CO2} from 5% to 9% (CO_2 levels within normal physiological limits). Later, it was found that a majority of serotonergic neurons (73%) in rat medulla tissue culture are highly chemosensitive to physiologically relevant changes in pH, and the same response occurs in isolated rat medullary slices (Wang *et al.* 2001). Cellular responsiveness persists following blockade of glutamate and GABA, indicating that chemosensitivity in serotonergic neurons is independent of input from glutamatergic neurons and GABA-ergic neurons in both tissue cultures and medullary slices, and is thus an intrinsic property of the cell (Richerson 1995; Wang and Richerson, 1999; Bradley *et al.* 2002). In fact, the intrinsic chemosensitivity of serotonergic neurons has been found to be greater than that observed for the other chemoreceptor candidates (Putnam *et al.* 2004). There is some evidence that serotonergic neurons also demonstrate chemosensitivity *in vivo*. A group of serotonergic neurons in the medulla of awake, behaving cats increase their firing rate by 160% in response to inhalation of 8% CO_2 (Veasey *et al.* 1995). When rat medullary raphe neurons are exposed to focal acidification *in vivo*, ventilation increases (Bernard *et al.*1996). It should be noted that although it is likely that chemosensitive neurons are sensitive to changes in pH and stimulate breathing, they also might be inhibited by acidosis and in turn release respiratory output from tonic inhibition (Wang *et al.* 2001). Whatever the mechanism, a large number of experiments have revealed the chemosensitive nature of serotonergic neurons. As such it is proposed that serotonergic neurons possess biologically important chemosensitivity both *in vitro* and *in vivo* (Richerson *et al* 2001).

The aforementioned studies demonstrated that serotonergic neurons may play a role in sensing changes in pH. However, it must be noted that "central" chemoreceptors can have diverse and wide-ranging effects, not just modulation of breathing. Serotonergic neurons may have an impact on other systems, like cerebrovascular control (Underwood *et al.* 1995, Cohen *et al.* 1996), arousal (Saper *et al.* 2001), and anxiety (Hendricks 2003). Richerson (2004) suggested that the serotonin system plays a global role in controlling pH homeostasis, motor control, and neural tissue homeostasis. Maintaining adequate ventilation is a small part of maintaining homeostasis, but a vital role nonetheless. Researchers have therefore focused on defining how serotonin is linked to ventilation and have found anatomical links that reveal an intimate physical connection between serotonergic neurons and the respiratory network (Richerson 2004).

1.3.2 Location

In order for serotonergic neurons to be central chemoreceptors that modulate breathing, they must 1) occur in appropriate regions where sensitivity to pH and/or CO_2 is necessary, and 2) have appropriate anatomical connections to affect respiratory output. Serotonergic neurons are indeed located in an optimal position to monitor blood pH levels. They are located in a region of the medulla containing large arteries and few veins. Blood within these regions has recently exchanged gas at the lungs and has not been greatly influenced by local tissue metabolism. Within these regions, distinct populations of serotonergic neurons occur in close proximity to arteries supplying the

brainstem including large branches of the basilar artery (Bradley *et al.* 2002; Severson *et al.* 2003; Messier *et al.* 2004). These neurons are proximal to blood vessels, allowing them to monitor the acid-base status of blood (Richerson *et al.* 2002). Serotonergic neurons in these positions have been shown to increase their firing rates in response to acidosis, as would be expected of a chemoreceptor (Richerson 2004).

1.3.3 Connectivity

If serotonergic neurons are important respiratory chemoreceptors, they must somehow influence ventilation after sensing changes in pH or CO_2. Serotonergic neurons possess the appropriate neuronal connections that would be expected of a chemoreceptor. Although not directly responsible for altering homeostatic processes, serotonergic neurons project to various regions of the brain that have direct roles in regulating a number of homeostatic processes. Serotonergic neurons project to areas of the brain that respond to changes in CO_2 and regulate a variety of functions, including arousal, anxiety, cardiovascular control, neural tissue homeostasis, and motor control. Specifically, serotonergic neurons project to medullary respiratory neurons innervating the diaphragm (Richerson 2004). Thus, they are able to monitor pH and/or CO_2 and transmit this information to appropriate targets in the brainstem respiratory network that affect breathing accordingly.

1.3.4 Serotonergic neuromodulation

Serotonergic neurons are capable of influencing respiratory patterns. Most serotonergic neurons in the brain are located within the midline raphe nuclei (Jacobs and Azmitia 1992), and a subset project to medullary respiratory neurons (Richerson 2004). This connection enables chemosensitive serotonergic neurons to modify the ventilatory pattern in response to changes in pH/CO_2. Several studies have shown that serotonin plays a key role in the response to increased CO_2; normally, ventilation in rats increases in response to induced acidosis in the medullary raphe (Feldman *et al.* 2003). However, Messier *et al.* (2004) found that selective lesioning of rat serotonergic medullary raphe serotonergic neurons decreases the ventilatory response to systemic CO_2. Along the same lines, destroying serotonergic neurons with saporin conjugated to an antibody to the serotonin transporter (SERT) reduced the ventilatory response to CO_2 *in vivo* (Nattie *et al.* 2004; Richerson, 2004). The loss of serotonergic modulation documented in these studies resulted in a diminished respiratory response to CO_2, implying that serotonin is required for an appropriate response to hypercapnia. Many serotonergic neurons are located close to penetrating arteries in a position appropriate for monitoring CO_2 in blood that will supply the central nervous system (Wang *et al.* 2002). Although not directly involved in regulation of respiratory muscles, serotonergic neurons project to a diverse array of brainstem and midbrain regions that directly modulate the respiratory network (Nattie *et al.* 2004). These and related studies serve as evidence that a sub-population of serotonergic neurons functions as important chemoreceptors.

1.4 5-HT_{1A} receptors

The 5-HT_{1A} receptors in areas of the brainstem, such as the midline raphe nuclei, that are proposed to act as central chemoreceptors, have been classified as somatodendritic autoreceptors occurring on the cell body (for review see Glennon *et al.* 2000). Similar receptors, $5HT_{1B/D}$, are classified as presynaptic autoreceptors and are located on the presynaptic membrane of neurons that synthesize and release serotonin (Pauwels 1997). Binding to 5-HT_{1A} autoreceptors in the raphe nuclei results in inhibition of serotonin neuron activation, while binding to $5HT_{1B/D}$ receptors suppresses release of neurotransmitter from the serotonergic neuron. The net result of 5-$HT_{1A\text{ and }B/D}$ autoreceptor binding is a reduction of serotonergic excitation of neurons that receive input from serotonin neurons. This study utilized a strong agonist for 5-HT_{1A} and 5-$HT_{1B/D}$ receptors to reduce or abolish post-synaptic influences, in order to demonstrate the role normally served by serotonergic neurotransmission. The prototypical agonist for 5-HT_{1A} and 5-$HT_{1B/D}$ receptors is the 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT), an aminotetralin derivative that binds 5-HT_{1A} and 5-$HT_{1B/D}$ receptors with high affinity (EC_{50} = 6.9, 125nm for 5-HT_{1A}, Del Tredici *et al.* 2004). 8-OH-DPAT is commonly used to reduce or abolish release of serotonin at neuron terminals. Although multiple receptor subtypes remain available for binding on postsynaptic neurons, administering the selective agonist 8-OH-DPAT to 5-HT_1 receptors in the midline raphe should bind to presynaptic 5-$HT_{1A/B}$ receptors with such an affinity as to be sufficient to prevent serotonergic chemoreceptive neurons from signaling (Del Tredici *et al.* 2004).

1.5 Chemoreceptor Controversy

In large part, the respiratory network is, by itself, insensitive to changes in pH. Despite ample *in vitro* evidence suggesting that serotonergic neurons could act as central chemoreceptors that modulate the respiratory network, there is some dispute about whether serotonergic neurons function *in vivo* as primary central chemoreceptors that activate the respiratory network. Guyenet *et al.* (2005) proposed that glutamatergic neurons of the retrotrapezoid nucleus (RTN) have a more robust response to pH than other neuron types. Unfortunately, this conclusion was drawn from experiments that utilized large changes in pH (7.5 to 6.9) that may not be relevant to the small changes typically seen in respiratory control (7.4 to 7.2), as described by Wang *et al.* (2002) and Putnam *et al.* (2004). Based on observations of anesthetized rats, Guyenet (2004) argued that hypercapnia has a marginal stimulatory effect on only a small percentage of medullary raphe serotonergic neurons *in vivo*. However, the "small percentage" of serotonergic neurons was actually 22%, and it is possible that a large part of those neurons project to respiratory neurons (Richerson 2004).

Based on data derived from anesthetized animals, Guyenet *et al.* (2005) argued that glutamatergic neurons of the retrotrapezoid nucleus are central chemoreceptors and serotonergic neurons are not. Guyenet and colleagues cite studies by Veasey *et al.* (1995) and Mulkey *et al.* (2004) who found that glutamatergic neurons in the ventral medulla altered firing rates, when acidified, and that glutamatergic disruptions altered chemosensitivity in anesthetized rats, while only a small minority of the serotonergic neurons they tested were stimulated when CO_2 increased (Mulkey *et al.* 2004).

The degree of chemosensitivity is an important factor in determining the relevance of a neuron type to respiratory control (Richerson 1998, Putnam *et al.* 2004, Guyenet *et al.* 2005). RTN neurons have a chemosensitivity index of 200% (Putnam *et al.* 2004) which is indeed larger than some other candidates for respiratory chemoreceptors (Putnam *et al.* 2004) but serotonergic neurons have a chemosensitivity index of 300% (Wang *et al.* 2002; Richerson 2004). Serotonergic neurons cannot be discounted in favor of glutamatergic neurons: serotonergic cells demonstrate a robust response to physiologically relevant levels changes in pH (Richerson 2004).

Three groups studying chemosensitivity agree that a major criterion for a chemoreceptor candidate, besides the degree of chemosensitivity, is that chemosensitivity is an intrinsic property (Richerson 1998, Putnam *et al.* 2004; Guyenet *et al.* 2005). It has not been proven that glutamatergic neurons of the RTN are intrinsically chemosensitive: studies by Mulkey *et al.* (2004) showed that RTN neurons were stimulated by changes in pH, but Richerson (2005) argued that the particular *in vivo* approach could not determine whether the response was intrinsic to the RTN cells or synaptically stimulated by other cells releasing neurotransmitters that could include serotonin. Glutamatergic neurons could not be considered primary chemoreceptors if they rely on phasic synaptic inputs to respond to changes in pH. On the other hand, serotonergic neurons have been shown to be intrinsically sensitive to changes in pH (Veasey *et al.* 1995; Wang *et al.* 1998; Wang and Richerson 2000), and, since they are intimately connected to the respiratory network, they are superior candidates for central respiratory chemoreceptors (Richerson 2004).

1.6 Hypothesis

Previously, only a small number of recordings had been made from unanesthetized, intact animals and only from a restricted subset of serotonergic neurons (Richerson 2005). Other research on serotonergic neurons, like the acidification studies by Wang *et al.* (2001), used *in vitro* or *in situ* methods, and the function of serotonergic neuromodulation *in vivo* remained unclear. The present study is designed to fill the gap and to determine the role of serotonergic neurons in chemosensitivity in an unanesthetized *in situ* preparation believed to exhibit patterns of nerve activity and chemosensitivity akin to breathing *in vivo*.

Serotonergic neurons meet the criteria to be effective chemoreceptors: they are sensitive to CO_2 *in vitro* and *in vivo*, they are located in appropriate anatomical positions to sense changes in pH levels, and they are connected to a variety of other brain regions that participate in respiratory responses (Richerson 2004). Serotonergic neurons possess characteristics *in vitro* and *in vivo* that would make them effective chemoreceptors, and their role as such was investigated in this project. By inhibiting serotonergic neuronal firing via stimulation of the 5-HT_{1A} somatodendritic autoreceptor, the present study explored the hypothesis that serotonergic neurons are critical for respiratory chemosensitivity.

Materials and Methods

2.1 Perfused rat brainstem preparation

The perfused rat brainstem preparation has been well characterized and is widely accepted as an appropriate tool for studies of the neural control of breathing (Paton 1996; St. John and Paton, 2000; Harris and St. John, 2003). The preparation is particularly desirable for the proposed investigations as it has many of the advantages of both *in vivo* and *in vitro* preparations. The preparation is without a cerebrum but otherwise the central nervous system is intact (Figure 1). Thus, the entire brainstem respiratory control network and associated tonic inputs are in place and not influenced by anesthesia beyond the initial dissection (Paton 1996). The preparation exhibits respiratory patterns of discharge on nerves innervating respiratory muscles, and it exhibits changes in these patterns associated with CO_2 exposure, comparable to breathing and ventilatory responses of the intact animal (Paton, 1996; St. John and Paton, 2000). Respiratory-modulated neuronal activities with discharge patterns similar to those *in vivo* also can be recorded in the medulla and pons and can be maintained for long durations. The preparation facilitates specific control of drug concentrations when bath-applied in the perfusate. It preserves a stable cardiovascular system during severe manipulations, as aortic perfusion pressures and flow may be held constant or regulated as an experimental variable (Paton 1996).

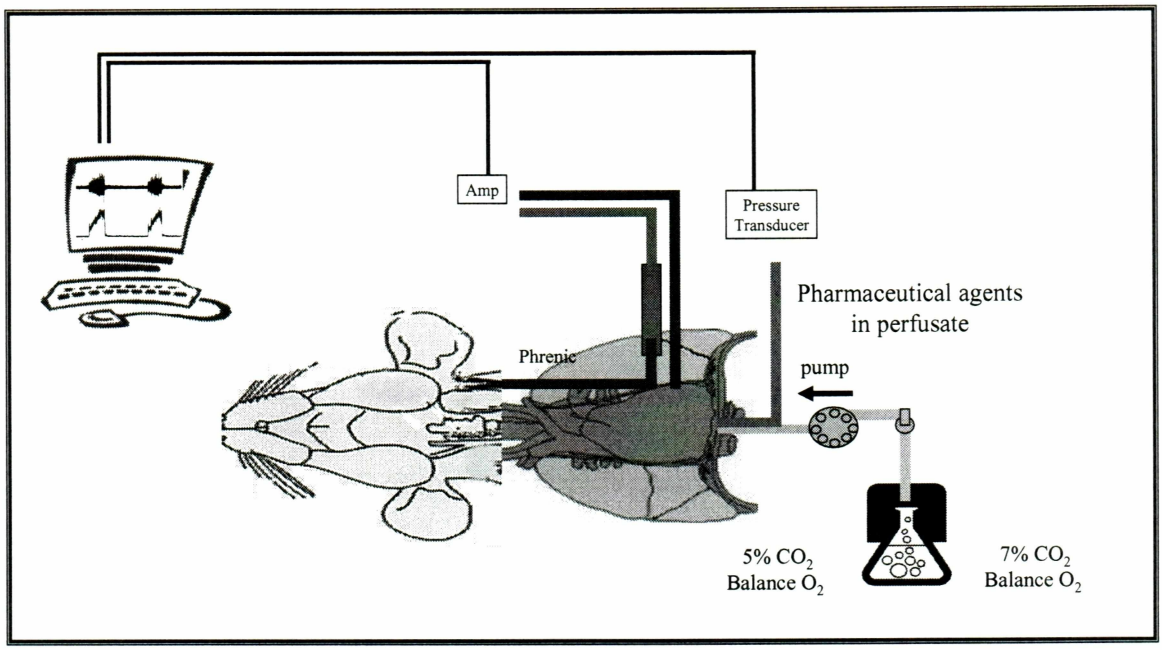

Figure 1. The perfused rat brainstem preparation, a well-characterized and widely accepted model for the neural control of breathing. The portion of the body caudal to the diaphragm is removed, and a catheter pumping artificial cerebrospinal fluid is inserted into the aorta. The perfusate passes from the reservoir, where it is equilibrated with varying levels of O_2 and CO_2, through a roller pump, filter, and bubble trap while perfusing the preparation at a pressure of ~50mmHg. 5% CO_2 is considered normocapnia and 7% CO_2 is hypercapnia. Phrenic activity is monitored by a bipolar electrode connected to the phrenic nerve; activity is amplified, filtered, integrated and recorded. Phrenic neurograms are recorded by the PowerLab data acquisition system.

This study employed juvenile male Simonsen S/A albino rats, *rattus novegicus* (Simonson Laboratories, Gilroy, CA). Rats were 24-27 days and weighed 80-120g. At this age the response of rats to hypercapnia has reached maturity (Stunden *et al.* 2001) For ease of handling, rats were anesthetized with halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) in a glass chamber until unresponsive. Rats were injected with an anticoagulant, 1.5ml heparin sodium injection (1000USP, Baxter, Deerfield, IL), and allowed to recover from anesthesia. After a fifteen minute period, rats were again anesthetized with halothane until spontaneous respiratory movement ceased (1-2 min). Following procedures outlined in the original description of this preparation (Paton 1996) the skull was opened at the coronal suture and all of the brain rostral to the colliculi removed by aspiration. The portion of the body caudal to the diaphragm was removed. The temperature of the remaining portion of the body was reduced by immersion in chilled artificial cerebrospinal fluid (aCSF; see below). The pelt was removed from the torso. The diaphragm was gently separated from the outer body wall using forceps and hemostats and the outer body wall was cut away, leaving a 3-4cm length of spinal cord. The heart, lungs and diaphragm were left intact. The descending aorta was freed from other tissue using hemostats and tweezers, and the end cut cleanly with surgical scissors. The right phrenic nerve was isolated and sectioned at the level of the diaphragm and attached to a bipolar glass capillary suction electrode.

Rats were positioned supine in a plastic, rectangular tray, and a catheter (French double lumen, 1.25mm, Braintree Scientific, Braintree, MA) was inserted into the aorta, advanced approximately 2cm rostrally, and tied in place with braided waxed silk (CS

surgical suture). One lumen of the aortic catheter was connected to a peristaltic pump delivering aCSF, equilibrated with 95% O_2-5% CO_2 and at an initial temperature of 30°C. The second lumen of the catheter was used to measure pressure within the aorta, which was maintained between 50-70mm Hg by regulating the speed of the perfusion pump. Gallamine triethiodide is added to the perfusate to eliminate motor movements (St. John and Paton 2000). The temperature of the brainstem was maintained at 29-31°C by adjusting the temperature of the perfusate entering the aorta via the heat exchanger system, and monitored constantly with a digital thermometer. Although slightly hypothermic, this temperature greatly enhanced the viability of the preparation, which, at 29-31°C, exhibits phrenic burst pattern frequencies equal to the breathing frequencies of hypothermic rats *in vivo* (St. John and Paton 2000).

The aCSF perfusate contained the following in distilled water (in mM): $MgSO_4$ (1), NaH_2PO_4 (1.25), KCl (4), $NaHCO_3$ (24), NaCl (115), D-glucose (10), $CaCl_2$ (2), Ficoll 70 (0.1785). Ficoll was used as an osmotic agent in perfusate solutions to replace non-ionic osmotically active particles, such as proteins. From a reservoir where the solution was equilibrated with O_2 and CO_2, the perfusate passed through a roller pump, heat exchanger, filter (Millipore 45uM), a "bubble trap", and then through the cannula in the aorta. There typically were small fluctuations in perfusion pressure (3-5mm Hg) resulting from the roller pump. Perfusate leaked from sectioned vessels in the animal and collected in a reservoir. The initial 50ml of perfusate was discarded to eliminate blood, the rest was recirculated.

2.2 Recording of neural activity

Phrenic nerve activities were monitored by bipolar electrodes. Glass capillaries were pulled to a fine taper and ground back to produce a needle with inner tip diameter of 30 microns, approximating the diameter of the phrenic nerve. Capillaries were held in a micromanipulator (Model KITE-R, WPI, Sarasota, FL) via a suction electrode connector (A-M Systems, Sequim, Washington). Perfusion pressure was gradually increased in increments of 10mm Hg approximately once every minute until phrenic activity returned with a eupneic, ramp-like pattern (Figure 2) (see also St. John and Paton 2000). Phrenic activity was monitored by the electrode, amplified (1^{st} stage DAM 50 differential amplifier, WPI, Sarasota, FL; 2^{nd} stage Model 5900 Universal amplifier, Gould instruments, Cleveland, OH); filtered (0.6-6.0 KHz) and integrated (50ms moving average) using full wave rectification (Model, CWE Incorporated, Wood Dale, IL). Raw and integrated signals were recorded to a computer data-acquisition system sampling at 200 Hz (Chart, Version 5.0, PowerLab, ADInstruments, Colorado Springs, CO). An oscilloscope and audio monitor were utilized intermittently to assist in the discrimination of phrenic burst signals. Data acquisition included tracings of raw and integrated phrenic neurograms (Figure 2), as well as continuous pressure and CO_2 measurements.

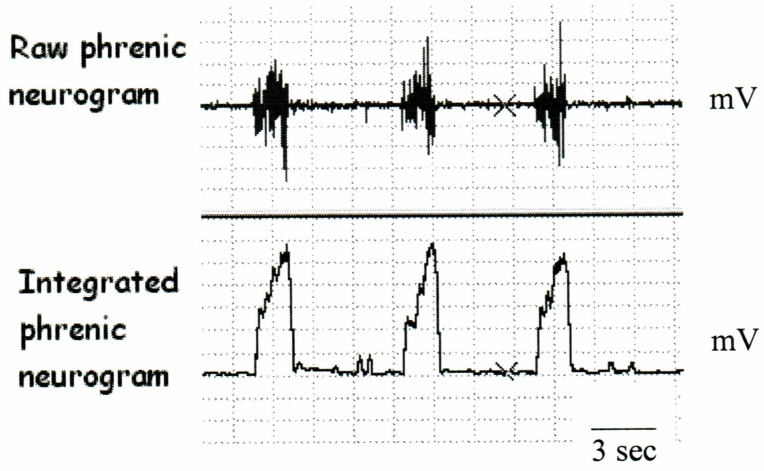

Figure 2. Steady phrenic neurogram burst pattern during normocapnia (5% CO_2). The raw phrenic neurogram, above, is integrated using a 50msec time constant and full-wave rectification to produce the integrated phrenic neurogram, below. The ramp-like rise of integrated activity from the phrenic nerve is consistent with eupnea ("normal breathing") *in vivo*.

Subsequent data analysis was conducted using the integrated signal and the data analysis utilities provided by the data acquisition system (PowerLab). Figure 3 illustrates the five burst pattern variables quantified from the integrated phrenic neurograms (Chart Data Analysis, Version 5.3): burst frequency (Fr), peak height (peak), time of neural expiration (period between bursts, Te), time of neural inspiration (burst duration, Ti) and area under the burst (area). The burst variables correlate with those measured in the first use of the perfused rat brainstem preparation by St. John and Paton (2000): neural inspiration corresponds to Ti, neural expiration is Te, Fr is calculated by Ti + Te/ 60 as an index of breathing frequency; peak height and area under the burst may serve either as indices of tidal volume, or the amount of air inhaled/exhaled, or both (St. John and Paton 2000). It was important to measure all five variables in the phrenic neurogram as they may change individually or in a coordinated manner (St. John and Paton 2000). It was unknown at the start of this study whether the inhibition of serotonergic modulation with 8-OH-DPAT would manifest as a change in one variable or more than one.

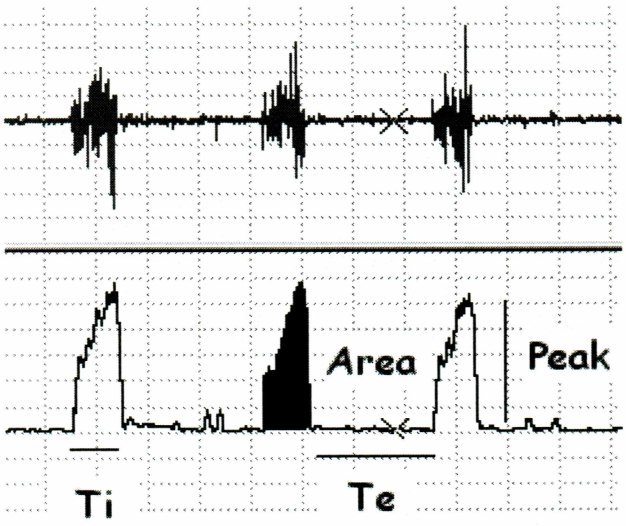

Figure 3. Phrenic nerve discharge during eupnea (normal breathing). Integrated activity of the phrenic nerve was quantified by measuring five burst pattern variables: peak height, Ti (time of inspiration), Te (time of expiration, between bursts), area under the peak, and burst frequency which is related to breathing frequency. Instantaneous frequency of bursts (Fr) was calculated as 60/ (Ti + Te).

2.3 Protocol

The preparation was allowed an initial equilibration period of at least 40 min, after temperature and pressure had equilibrated at appropriate levels (29-31°C, 50-70mm Hg). Under initial conditions, the perfusate was equilibrated with 95% O_2 -5% CO_2 (physiologically relevant levels of O_2 saturation). To mimic normocapnic/hypercapnic conditions, levels of O_2 and CO_2 were altered by changing the concentrations in the perfusate using manual gas flow regulators. CO_2 concentrations equilibrating with the perfusate were constantly monitored with a CO_2 gas analyzer (Ametek CD-3A, Paoli, PA). Pilot studies demonstrated that less than 5 min was necessary following a change in CO_2 levels to allow the perfusate to fully equilibrate and circulate to the experimental preparation. As such, measurements of the hypercapnic response were taken only in the second 5 min of a 10 min hypercapnic challenge (Figure 4).

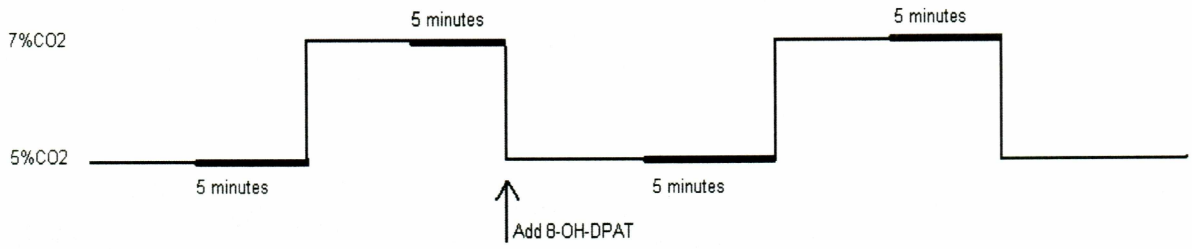

Figure 4. The experimental protocol. Measurements of the five burst variables were measured in the second 5 minutes of each ten minute increment, to allow the perfusate to equilibrate with 5% or 7% CO_2 during the first five minute increment. Bold lines indicate periods during the experiment during which normocapnic and hypercapnic responses were recorded. A baseline hypercapnic response in each animal was recorded. Doses of 0, 1.5, 3, and 6uM 8-OH-DPAT were applied to the perfusate, then the preparation was given a second hypercapnic (7%CO_2) challenge and its responses to hypercapnia were compared, before and after application of the 5-HT_{1A} agonist 8-OH-DPAT,.

2.4 Responses to hypercapnia

All preparations were exposed to one hypercapnic challenge to record a baseline hypercapnic response. The rats were exposed to hypercapnia by changing gas equilibrating in the perfusate from 5% to 7% CO_2. A 5-min period following the change in CO_2 was allowed for equilibration of the perfusate. The rats were then maintained at CO_2 of 7% for 5min, after which CO_2 was returned to 5%. A period of 10min was allowed for re-equilibration between subsequent changes in CO_2.

2.5 Responses to hypercapnia following serotonin block

In order to characterize the response of the respiratory system without serotonin modulation, 8-Hydroxy-2-(di-n-propylamino) tetralin hydrobromide, (8-OH-DPAT, Aldrich, St. Louis, MO), a high affinity 5-HT_{1A} receptor agonist, was added to the perfusate. This study focused specifically on the 5-HT_{1A} receptor subtype because it provided a tool for modification of the serotonergic system by inhibiting the presynaptic serotonin neurons. 5-HT_{1A} receptors are located primarily on the presynaptic cell soma and at the presynaptic cleft. Blockade of these receptors inhibits serotonin synthesis and release from the presynaptic neuron (Pauwels 1997). In this way, pharmacological activation of 5-HT_{1A} receptors reveals the importance of serotonergic neuromodulation.

After an initial hypercapnic challenge, prior to drug application, the preparation was allowed to equilibrate with 5% CO_2. 8-OH-DPAT was added to the perfusate and the preparation was again allowed to equilibrate for 10min to ensure sufficient equilibration with the 8-OH-DPAT solution.

After the dose of 8-OH-DPAT was added into the perfusate reservoir, 10 min were allowed for equilibration of 8-OH-DPAT. Then the CO_2 was again increased from 5% to 7% for 10 min, and then reduced again to 5% for a 10 min recovery period. 8-OH-DPAT was administered in four doses: 0uM (sham treatment), 1.5uM, 3uM and 6uM. Doses of 0uM provided timed controls, and equivalent volumes of mock aCSF were added to the perfusate in place of the pharmacological agent.

2.6 Data analysis

Phrenic neurograms recorded in PowerLab were analyzed using Chart V.5.3 (PowerLab, ADInstruments). An analysis template program was developed that marked burst peaks on the phrenic neurograms and subsequently quantified five variables: Ti, Te, area, peak, and frequency (Figure 2). Values for the burst characteristics were determined from the integrated activity of the phrenic nerve. These included measurements of burst duration (neural inspiration, Ti), the period between bursts (neural expiration, Te), the area under the integrated curve of the burst (area), and the peak height of the integrated burst (peak). In addition, the instantaneous burst frequency (Fr) was calculated for each burst as 60/ (Ti + Te).

The neural ventilatory response to hypercapnia was evaluated at four different doses of 8-OH-DPAT (0, 1.5, 3, 6uM). The addition of 9uM 8-OH-DPAT greatly disrupted burst pattern, such that burst variables and their change with CO_2 exposure could not be adequately assessed. Responses were evaluated as the perfusate was changed from one equilibrated with 95% O_2-5% CO_2 (normocapnia) to one with 93% O_2-

7% CO_2 (hypercapnia). The variables were analyzed in four separate periods during each experiment: 1) 5 min of exposure to normocapnic perfusate immediately prior to hypercapnia, 2) 5 min of exposure to hypercapnic perfusate, 3) 5 min of exposure to normocapnic perfusate with 8-OH-DPAT, and 4) 5 min of exposure to hypercapnic perfusate with 8-OH-DPAT. During these four periods, burst pattern variables were quantified from the integrated phrenic neurogram. The burst pattern variables were assessed during normocapnia (5% CO_2) and hypercapnia (7% CO_2) to determine any changes in the burst characteristics associated with the normal hypercapnic response. The proportional change in each burst parameter during hypercapnia relative to the value during normocapnia was calculated. Values from the five burst variables were recorded to the PowerLab DataPad program and subsequently into a formatted Excel spreadsheet that quantified the hypercapnic response by calculating a ratio (hypercapnic value/ normocapnic value) under baseline conditions and following treatment with sham or doses of 8-OH-DPAT. Relative values equal to 1 are associated with no response, while values not equal to 1 represent a reduction or increase in the response to hypercapnia. Variables were assessed following 8-OH-DPAT treatment and compared with pre-treatment normocapnic values to determine the impact of serotonin neuron inhibition on normal burst pattern. Then, any changes in burst characteristics during hypercapnia, and the changes in burst pattern between normocapnia and hypercapnia, were compared before and after 8-OH-DPAT treatment to determine the impact of serotonin neuron inhibition on hypercapnic burst pattern responses.

Integrated activity of the phrenic nerve was quantified using Ti, Te, area, peak, and frequency as described above. Statistical evaluations (Kaleidagraph Version 4.0, Synergy Software, Reading, PA) of sham experiments were by the non-parametric Two-Way Repeated Measures ANOVA, with time and CO_2 level as factors, and post hoc analysis Tukey test. The response to hypercapnia and the drug response were evaluated using comparison of group means using a One-Way Repeated Measures ANOVA and post hoc Tukey test. A Two-Way ANOVA with Student-Neuman-Keuls post hoc comparison test was employed to establish whether or not there was a significant difference among the different doses of 8-OH-DPAT and whether it would be valid to pool across drug doses. Probabilities less than 0.05 were considered significant.

Results

3.1 Initial conditions

Under normocapnic conditions (5% CO_2, prior to 8-OH-DPAT application) phrenic burst discharge had a characteristic incrementing profile consistent with previous observations (St. John and Paton, 2000). Figures 2 and 3 illustrated representative patterns of discharge seen in the phrenic motor output of the preparation (St. John and Paton 2000). Phrenic bursts were characterized as a normal breathing (or "eupneic") pattern akin to phrenic discharge during normal spontaneous breathing *in vivo* (Figure 5). Under constant normocapnic conditions (without 8-OH-DPAT) eupneic phrenic burst discharge appeared stable over time and comparable between preparations. Time control trials confirmed that eupneic burst patterns during normocapnia and hypercapnia would remain consistent in preparations for periods in excess of four hours, which was longer than the time required for the experimental protocol (about two hours).

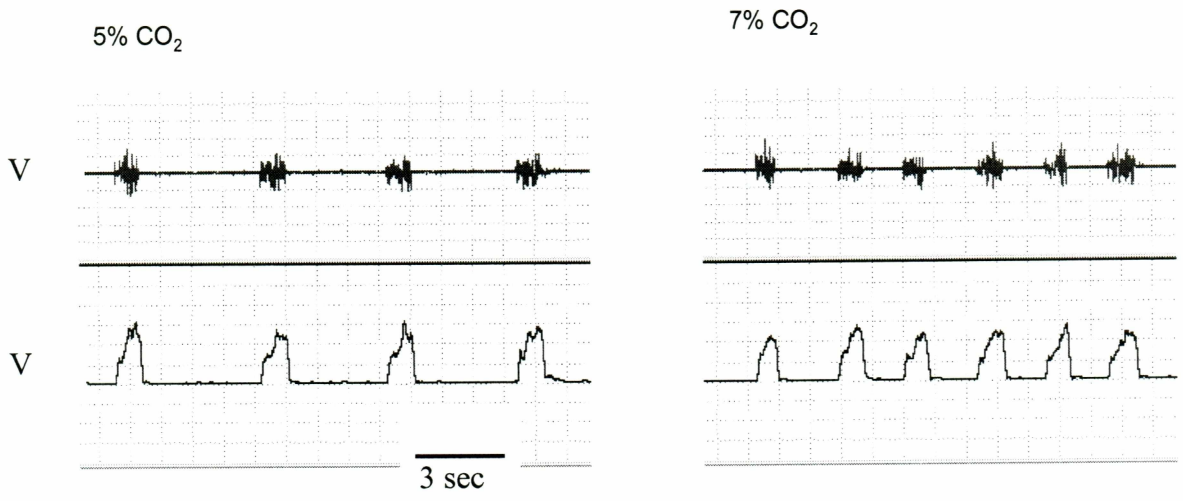

Figure 5. Representative phrenic neurograms from the perfused rat brainstem preparation. The neurograms demonstrate phrenic burst discharge under conditions of normocapnia (5%CO_2) and hypercapnia (7% CO_2). Note how the frequency of bursts in the integrated neurogram (bottom tracing) increases with hypercapnia but the other burst characteristics (Ti, Te, peak, area) do not.

On average, under initial conditions of 5% CO_2 (n=18), phrenic discharge had a Ti of 0.46 ± 0.02s (Figure 6), a Te of 4.05 ± 0.71s (Figure 7), an area of 0.39 ± 0.15V*s (Figure 8), and peak of 1.34 ± 0.34V (Figure 9). Bursts occurred with Fr of 22.9 ± 3.01 bursts per minute (Figure 10).

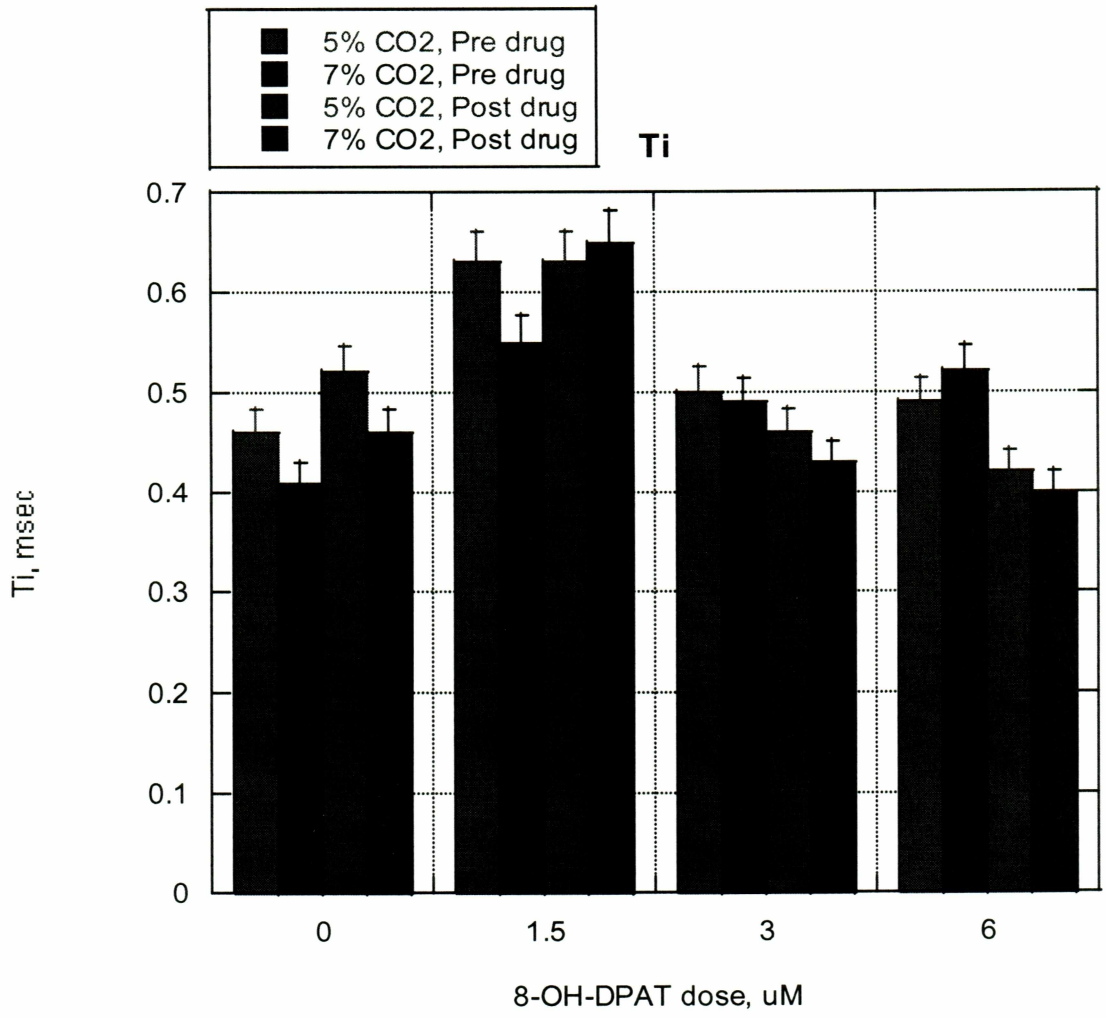

Figure 6. Values for the variable Ti (burst duration). Measurements were made under normocapnic and hypercapnic conditions, before and after application of the $5HT_{1A}$ receptor agonist 8-OH-DPAT. Data are mean ± SE for n=6 preparations.

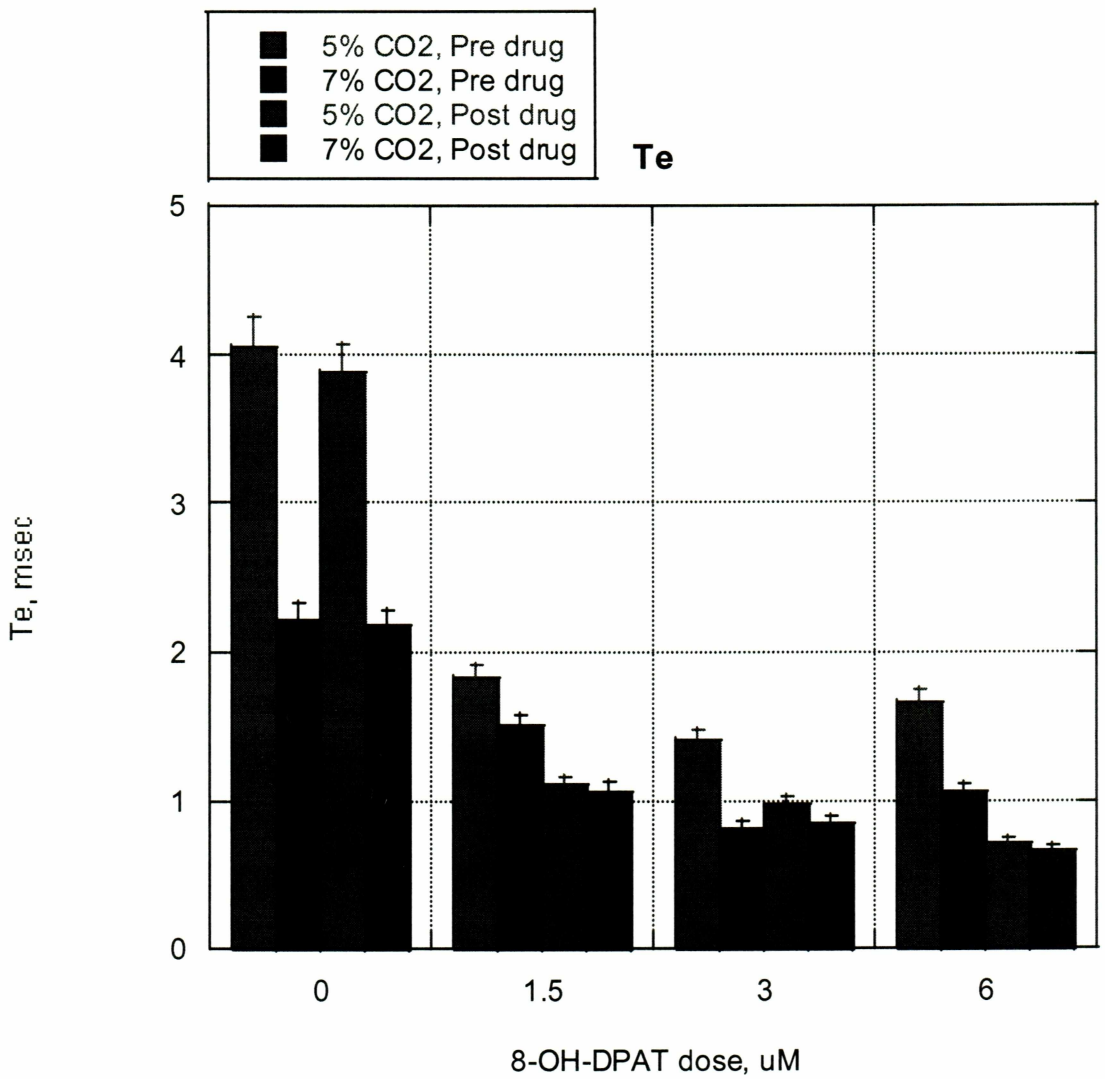

Figure 7. Values for the variable Te (neural expiration). Measurements of inter-burst pause under normocapnic and hypercapnic conditions, before and after application of the $5HT_{1A}$ receptor agonist 8-OH-DPAT. Data are mean ± SE for n= 6 preparations.

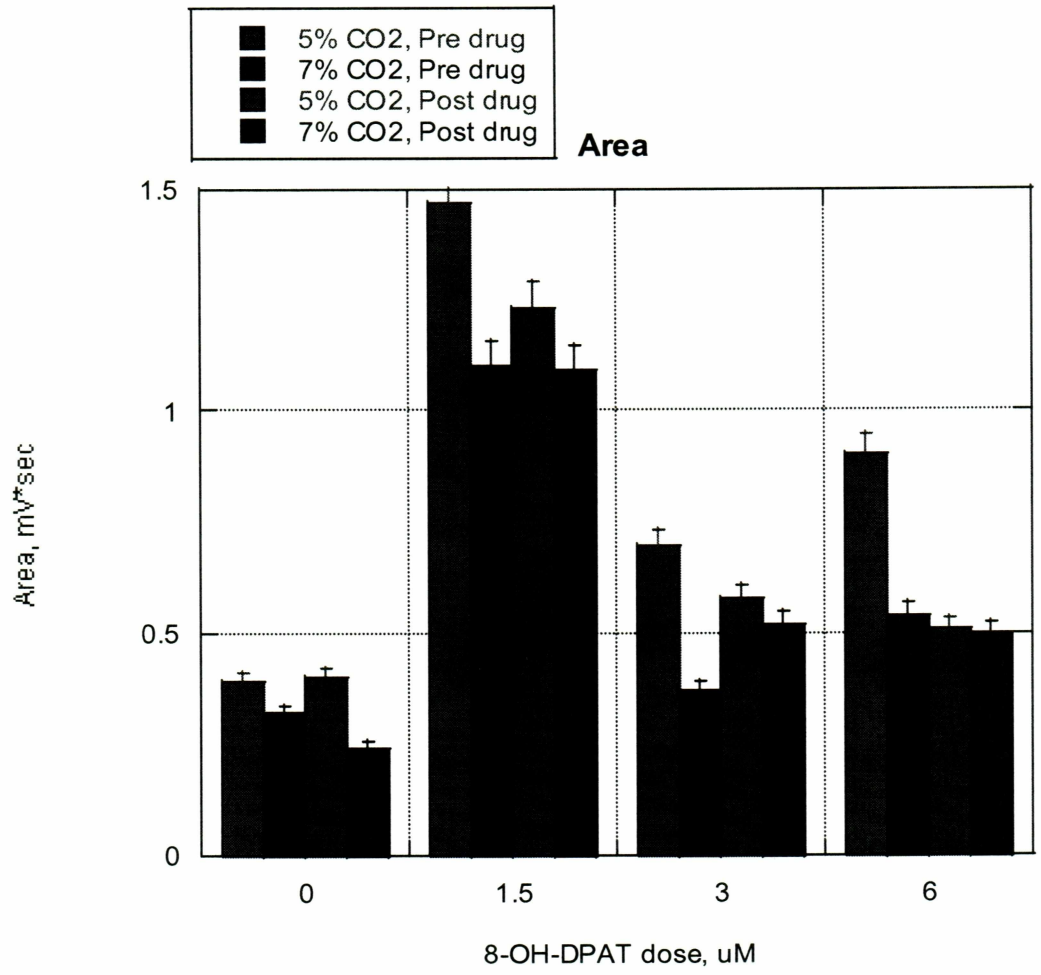

Figure 8. Values for the variable area. Measurements were made under normocapnic and hypercapnic conditions, before and after application of the $5HT_{1A}$ receptor agonist 8-OH-DPAT. Data are mean ± SE for n= 6 preparations.

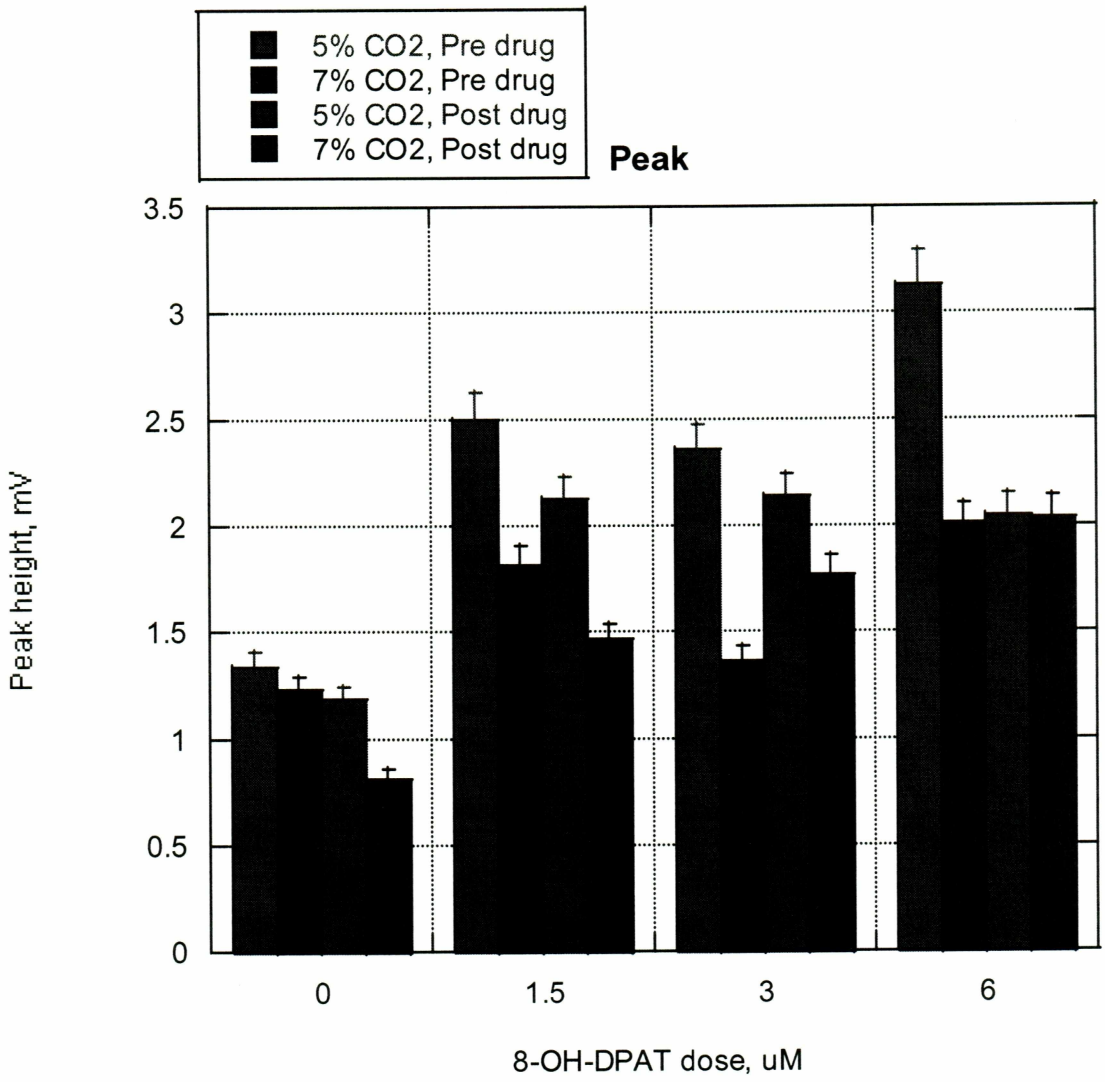

Figure 9. Values for the variable peak. Measurements of neural burst peak were made under normocapnic and hypercapnic conditions, before and after application of the $5HT_{1A}$ receptor agonist 8-OH-DPAT. Data are mean ± SE for n= 6 preparations.

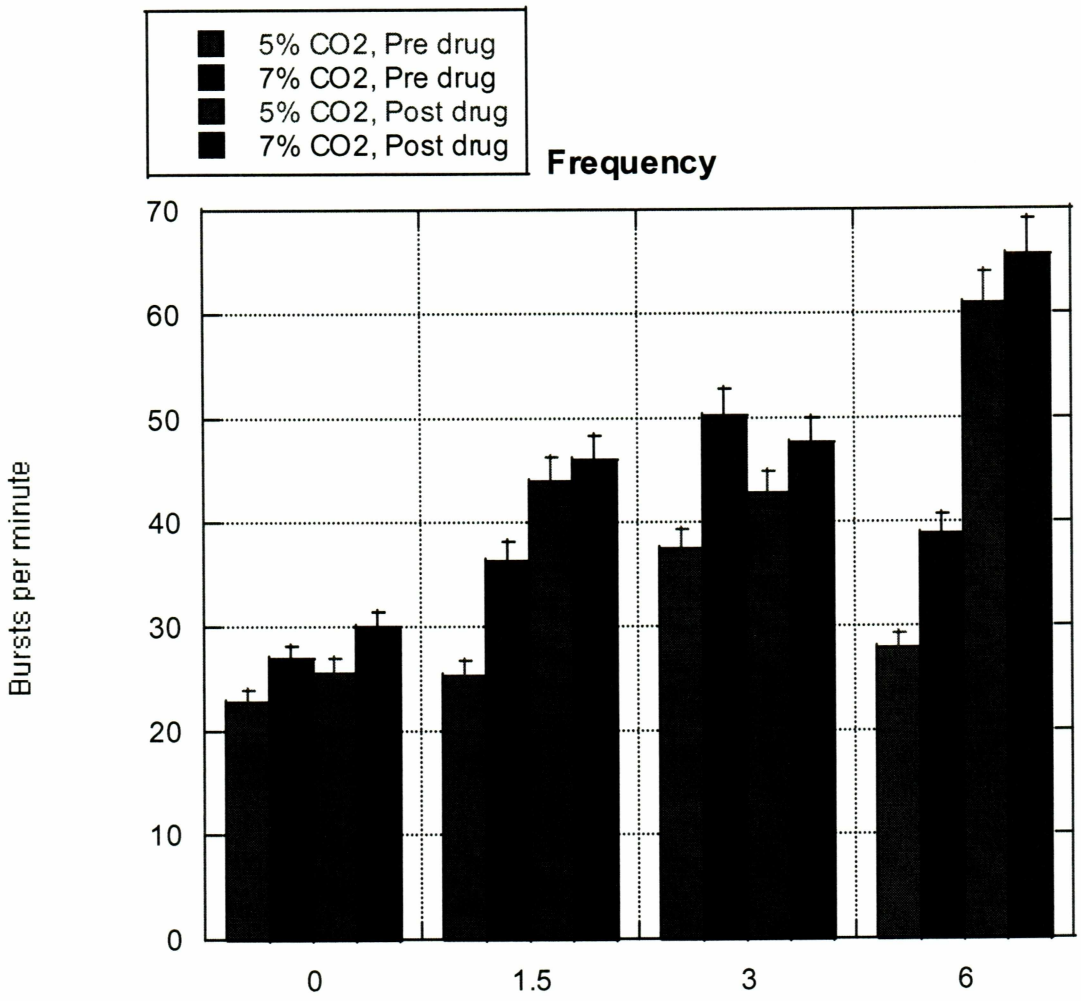

Figure 10. Values for the variable frequency (Fr). Instantaneous frequency of neural bursts was measured under normocapnic and hypercapnic conditions, before and after each application of the $5HT_{1A}$ receptor agonist 8-OH-DPAT. Data are mean ± SE for n= 6 preparations.

3.2 Hypercapnic response

During hypercapnia (7% CO_2, prior to 8-OH-DPAT application) phrenic discharge had an average (n=18) Ti of 0.41 ± 0.04s (Figure 6), Te of 2.21 ± 0.24s (Figure 7), an area under integrated burst of 0.32 ± 0.17V*s (Figure 8), and peak of 1.23 ± 0.33V (Figure 9). Bursts occurred with frequency of 26.9 ± 4.3 bursts per minute (Figure 10).

To quantify the hypercapnic response (before 8-OH-DPAT), burst variables were compared between periods of exposure to 5% and 7% CO_2 and expressed as a ratio (value at 7%/ value at 5%). These proportional values demonstrated the degree of change in the burst pattern following a hypercapnic challenge (a value of 1 indicates no change, a value greater than 1 indicates an increase in the quantity of the variable as CO_2 increased, and a value less than 1 indicates a decrease in the quantity of the variable as CO_2 increased).

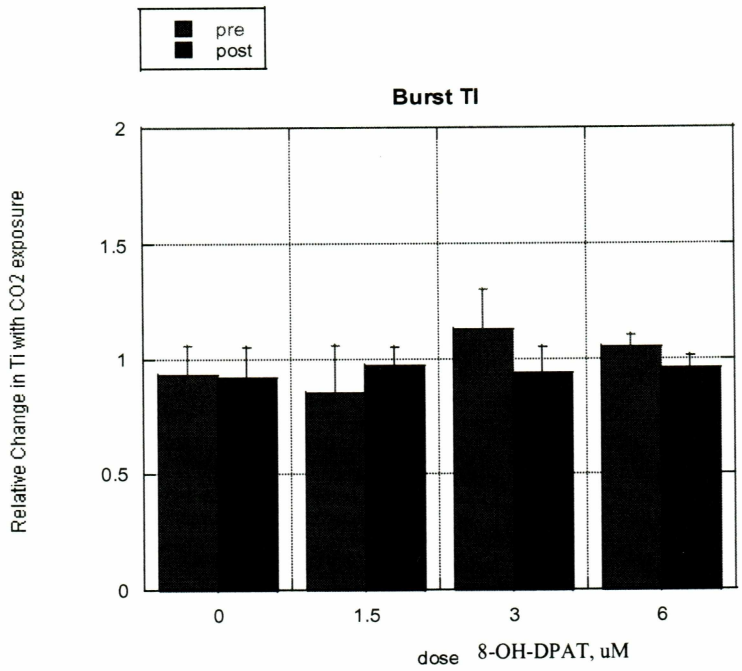

Figure 11. The relative change in the duration of the burst (Ti), with hypercapnia and drug. Measurements were made following an increase from 5% to 7% CO_2, pre- and post- treatment with doses of $5HT_{1A}$ agonist 8-OH-DPAT. Data are mean ± SE for n=6 preparations. A value of 1 implies no change, while values greater than 1 show an increase in Ti and less than 1 show a relative decrease in Ti. There is no statistically significant difference in Ti (p=1.0) during the normal hypercapnic response. The burst duration response did not significantly change after treatment with 8-OH-DPAT (p=0.821).

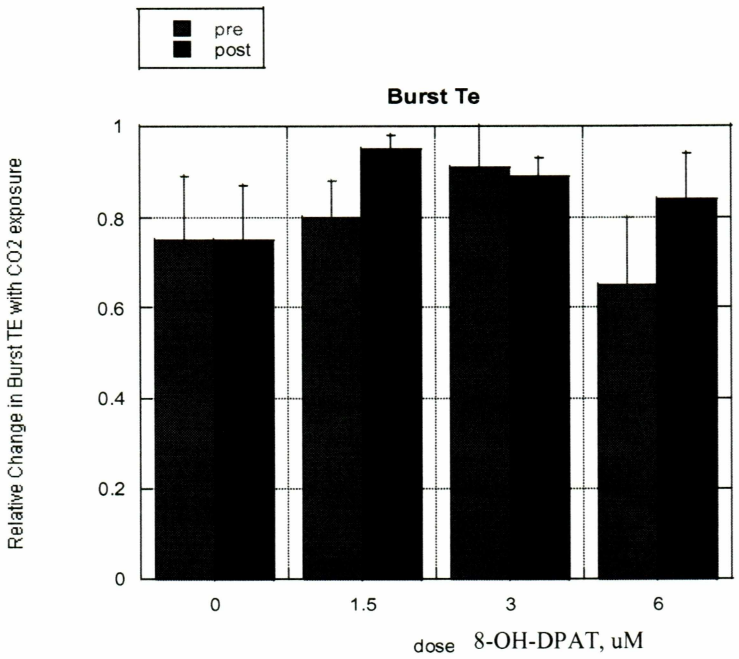

Figure 12. The relative change in the time between bursts (Te) with hypercapnia and drug. Measurements were made following an increase from 5% to 7% CO_2, pre- and post-treatment with $5HT_{1A}$ agonist 8-OH-DPAT. Data are mean ± SE for n=6 preparations. A value of 1 implies no change, while values greater than 1 show an increase in Te and less than 1 show a relative decrease in Te. There was a statistically significant reduction in Te ($p=<0.001$) during the normal hypercapnic response. There was not a statistically significant difference in Te after treatment ($p=0.073$).

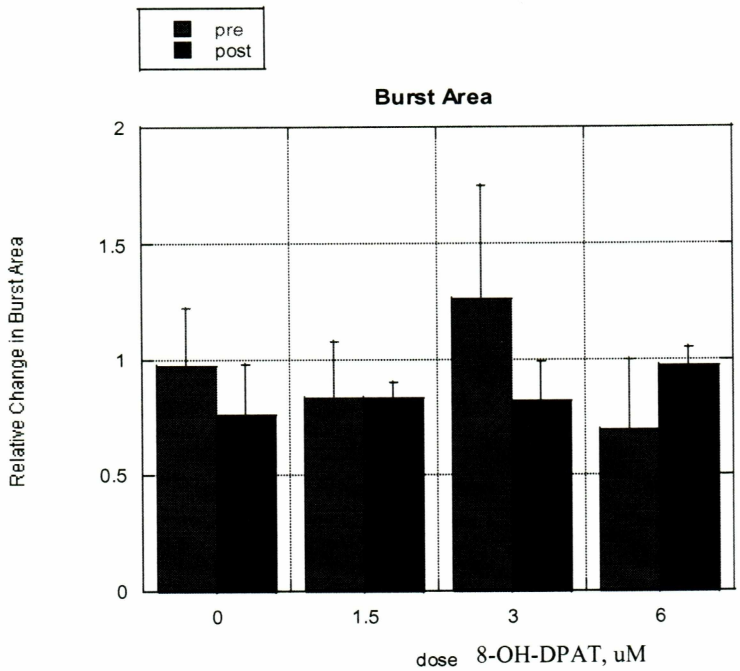

Figure 13. The relative change in burst area with hypercapnia and drug. Measurements were made following an increase from 5% to 7% CO_2, pre- and post-treatment with $5HT_{1A}$ agonist 8-OH-DPAT. Data are mean ± SE for n=6 preparations. There was not a statistically significant difference in area (p=0.637) during the hypercapnic response. A value of 1 implies no change, while values greater than 1 show an increase in frequency, and less than 1 show a relative decrease in frequency.

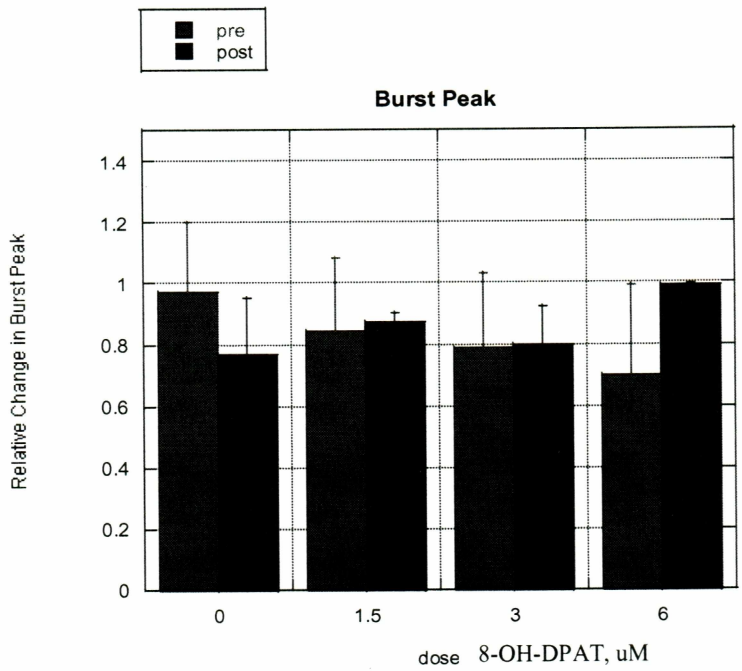

Figure 14. The relative change in burst peak with hypercapnia and drug. Measurements were made following an increase from 5% to 7% CO_2, before and after treatment with $5HT_{1A}$ agonist 8-OH-DPAT. Data are mean ± SE for n=6 preparations. A value of 1 implies no change, while values greater than 1 show an increase in peak height and less than 1 show a relative decrease in peak. There was no statistically significant difference in peak height (p=1.000) during the normal hypercapnic response. The burst peak height response was not significantly changed after treatment with 8-OH-DPAT (p=0.272).

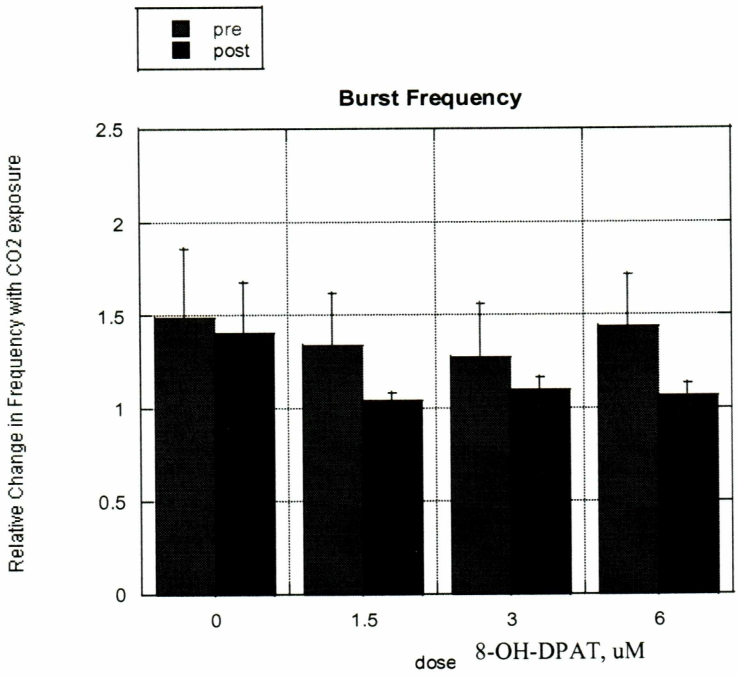

Figure 15. The relative change in burst frequency with hypercapnia and drug. Measurements were made following an increase from 5% to 7% CO_2, before and after treatment with $5HT_{1A}$ agonist 8-OH-DPAT. Data are mean ± SE for n=6 preparations. A value of 1 implies no change, while values greater than 1 show an increase in frequency, and less than 1 show a relative decrease in frequency. There was a statistically significant increase in frequency ($p=<0.001$) during the normal hypercapnic response. The frequency response was significantly reduced ($p= 0.018$) following treatment with 8-OH-DPAT, regardless of dose.

The only burst variables that changed significantly with hypercapnia were Fr and Te. A Repeated Measures ANOVA revealed a statistically significant increase in the frequency (Fr) response under hypercapnic conditions ($p=<0.001$) and a pairwise multiple comparison Tukey test confirmed this finding ($p<0.05$). Using the Repeated Measures ANOVA on ranks, with time and CO_2 level as factors, also showed a significant change in Te ($p=<0.001$) confirmed by a pairwise multiple comparison procedure (Tukey test, ($p<0.05$). These data indicate that the hypercapnic response was mediated by changes in the duration of neural expiration and burst frequency.

3.3 Time controls

There was no time-dependent response to CO_2 (St. John and Paton 2000). The initial values for a group of animals that received sham drug treatment (n=6) did not change significantly over time. Te, peak and area were unchanged. There were no significant differences in hypercapnic responses of any burst characteristic between initial conditions and following sham treatment. Using a Two-Way Repeated Measures ANOVA, values for burst area and burst peak were comparable to those seen in control animals (none showed a significant difference before and after CO_2 or over time). The frequency (Fr) response to hypercapnia was comparable to those seen under initial conditions. Frequency changed significantly($p=0.016$) with CO_2, as expected (St. John and Paton 2000) and the frequency response to hypercapnia remained statistically unchanged over time ($p=0.454$), before and after sham treatment. Unlike the trend

demonstrated by the pool of control animals (n=18), the values for Te in the smaller subset of sham-treated animals (n=5) was not changed with hypercapnia. The values for Te did not change significantly with time (p=0.827) or with hypercapnia (p=0.316). Additionally, there was no statistically significant change in Ti either with time (p=0.131) or with CO_2 (p=0.445). Despite no detectable changes in Te or Ti, the Fr response remained robust throughout repeated exposures to hypercapnia, and this response did not change over time.

3.4 Dose-dependent response

During pilot experiments, it became obvious that 1.5, 3 and 6uM 8-OH-DPAT were appropriate doses but that 9uM disrupted the baseline phrenic neurogram to such an extent that eupneic bursts were at times difficult to determine. As such, the 9uM dose was excluded from subsequent analysis (Figure 16).

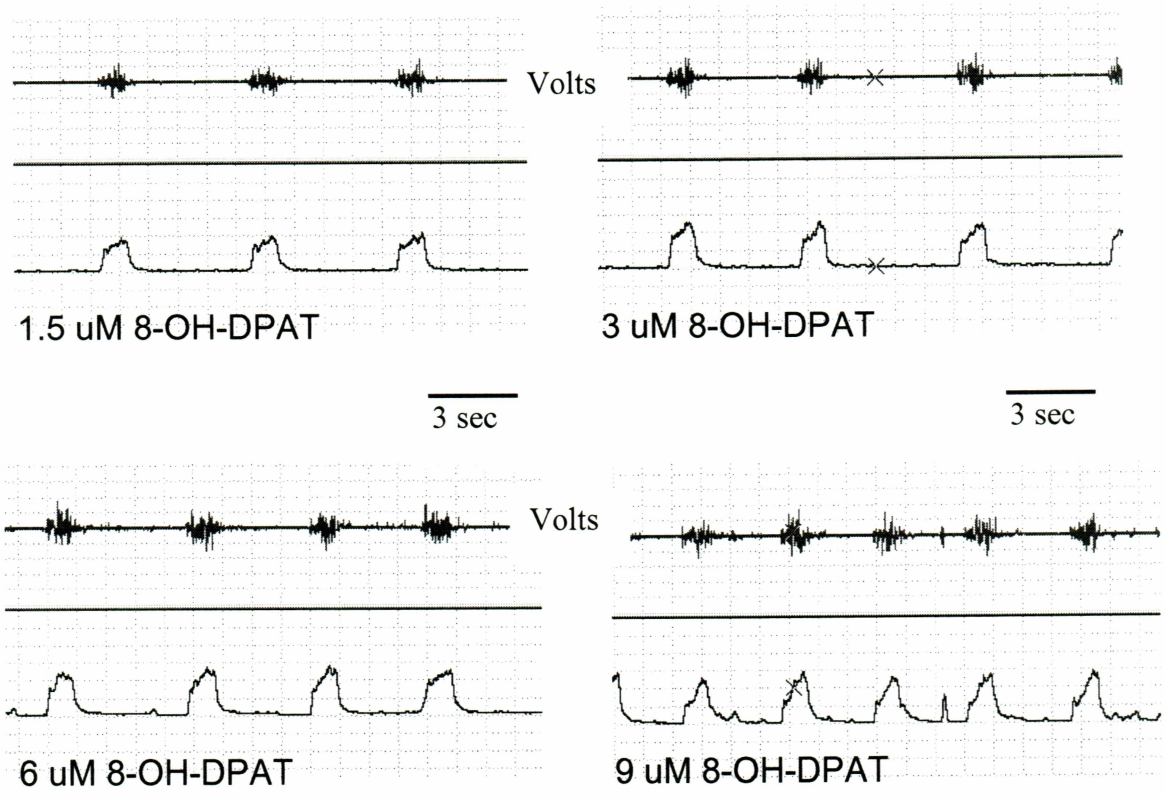

Figure 16. Typical phrenic burst patterns occurring after 8-OH-DPAT treatment. Phrenic neurograms are shown under normocapnic conditions, at doses of 1.5, 3 and 6uM, and the best possible example of burst pattern following a 9uM dose. In most cases, burst pattern was difficult to characterize with 9uM treatments. 9uM was determined to disrupt the baseline pattern to such an extent that it prohibited statistical analysis of the bursts; therefore doses of only 1.5, 3 and 6uM were used in further experiments.

Using a Two-Way ANOVA with time (pre-and post-drug) and dose as factors, we found that there were no significant changes with time or with drug dose in: area ($p=0.924$), peak (0.853), Te (0.996) or Ti (0.834), and no interaction between time and dose. However, there was a statistically significant difference in Fr before and after 8-OH-DPAT ($p=0.018$). Although this difference in Fr existed before and after treatment, there was no difference between the different doses (1.5, 3, and 6uM; excluding sham dose) either before or after 8-OH-DPAT treatment ($p<0.05$), establishing that pooling all data from all drug doses was statistically valid. All concentrations of 8-OH-DPAT used (1.5, 3, and 6 uM) were sufficient to fully saturate 5-$HT_{1A/B}$ receptors in the preparation (Del Tredici *et al.* 2004), establishing that pooling between doses is biologically valid.

3.5 Influence of 8-OH-DPAT

Following application of 8-OH-DPAT (pooled between 1.5, 3, and 6uM doses, n=13), the burst pattern variables were analyzed under normocapnic conditions. There were no significant differences in any of the variables between sham-treated animals and animals that received 8-OH-DPAT under normocapnic conditions (using a One-Way Repeated Measures ANOVA, comparing the values for Ti, Te, area, peak and Fr all presented $p>=0.05$). 8-OH-DPAT therefore did not affect the "eupneic" burst patterns at 5% CO_2. The perfusate was then changed from 5% CO_2 to 7% CO_2. Mean values for Ti, peak and area were statistically unchanged. When normalized to normocapnic (5% CO_2) values, the proportional changes in burst pattern variables with hypercapnia were:

Ti= 1.13 (Figure 16), Te= 0.9 (Figure 17), peak= 0.89 (Figure 18), area=1.02 (Figure 19), and Fr= 1.12 (Figure 20). (A change greater than 1 implies an increase in the burst pattern variable after hypercapnia; a change of less than one implies a relative decrease in hypercapnia). Of these proportional values, only frequency (Fr) and time between bursts (Te) differed significantly from 1. The normal increase in frequency (St. John and Paton 2000) with hypercapnia seen in untreated animals (see initial values section above) was abolished after application of 8-OH-DPAT ($p=0.028$) and a multiple comparison Tukey test confirmed this ($p=0.029$). 8-OH-DPAT abolished the increase in frequency (breaths per minute) response to hypercapnia.

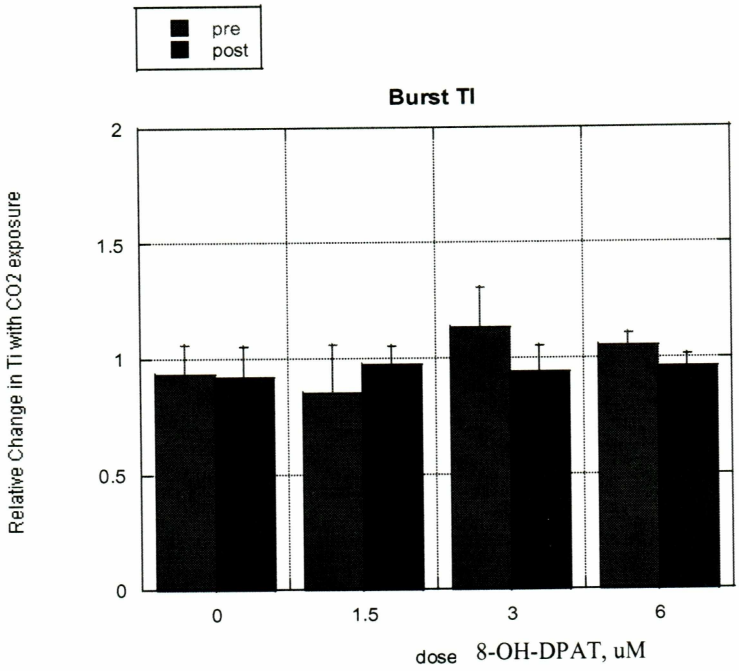

Figure 17. The relative change in the duration of the burst (Ti) following an increase from 5% to 7% CO_2, before and after treatment with $5HT_{1A}$ agonist 8-OH-DPAT. Data are mean ± SE for n=6 preparations. A value of 1 implies no change, while values greater than 1 show an increase in Ti and less than 1 show a relative decrease in Ti. There was no statistically significant difference in Ti ($p=1.0$) during the normal hypercapnic response. The burst duration response was not significantly changed after treatment with 8-OH-DPAT ($p=0.821$).

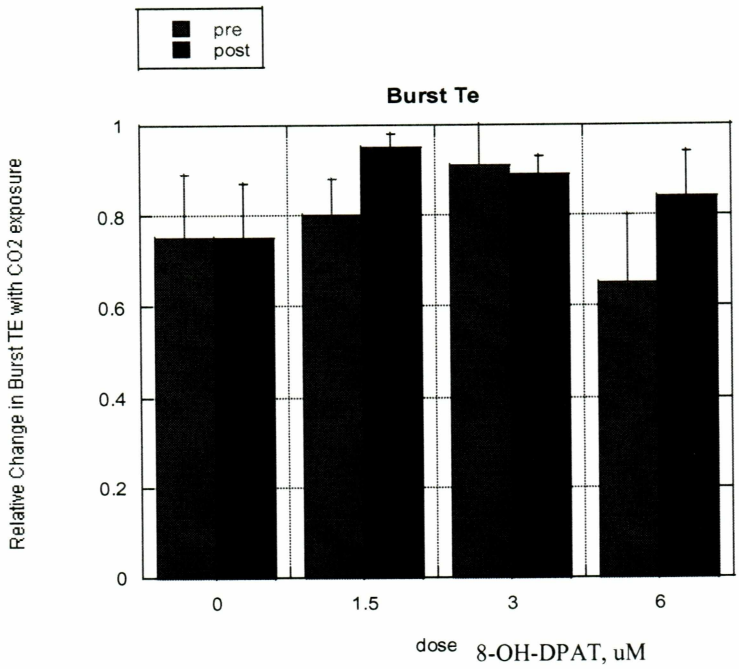

Figure 18. The relative change in the time between bursts (Te) following an increase from 5% to 7% CO_2, before and after treatment with 5HT-1A agonist 8-OH-DPAT. Data are mean ± SE for n=6 preparations. A value of 1 implies no change, while values greater than 1 show an increase in Te and less than 1 show a relative decrease in Te. There was a statistically significant reduction in Te ($p=<0.001$, n=6) during the normal hypercapnic response. There was not a statistically significant difference in Te after treatment ($p=0.073$).

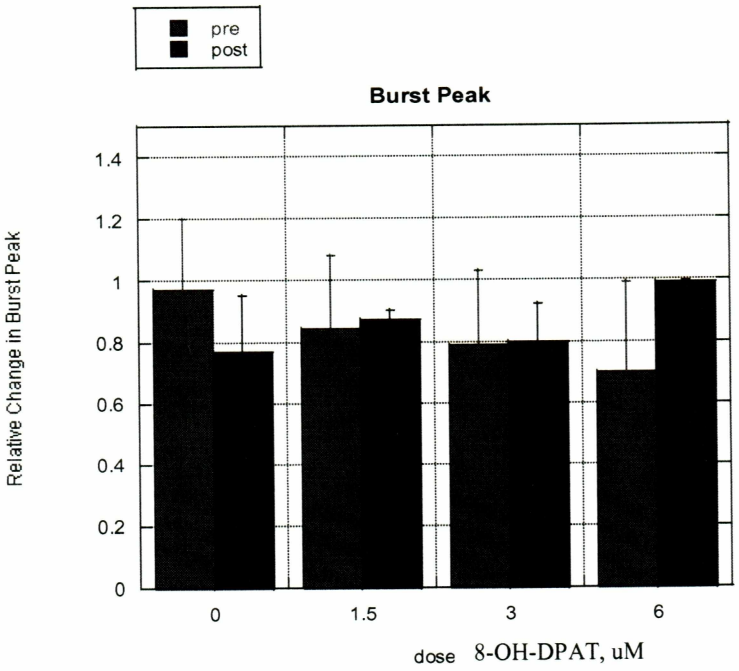

Figure 19. The relative change in burst peak (Peak) following an increase from 5% to 7% CO_2, before and after treatment with 5HT-1A agonist 8-OH-DPAT. Data are mean ± SE for n=6 preparations. A value of 1 implies no change, while values greater than 1 show an increase in peak height and less than 1 show a relative decrease in peak. There was no statistically significant difference in peak height (p=1.000, n=6) during the normal hypercapnic response. The burst peak height response was not significantly changed after treatment with 8-OH-DPAT (p=0.272).

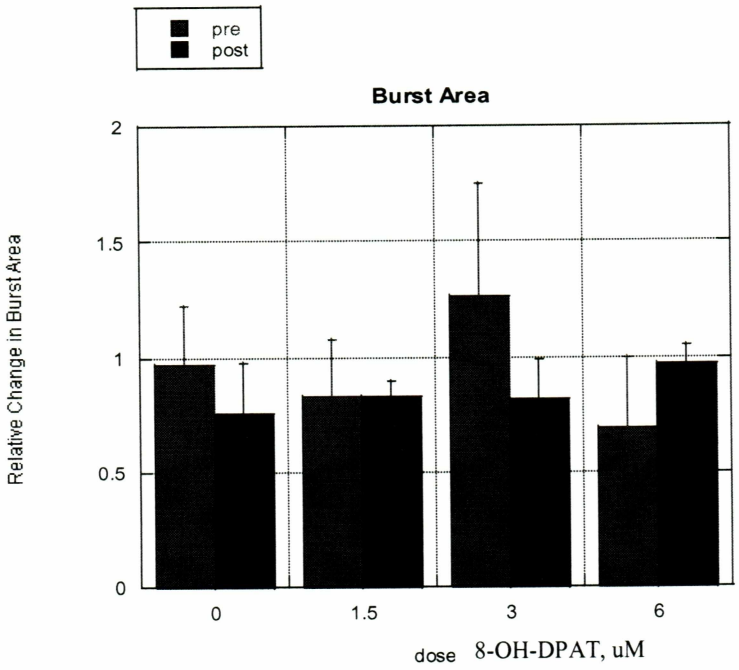

Figure 20. The relative change in burst area (Area) following an increase from 5% to 7% CO_2, before and after treatment with 5HT-1A agonist 8-OH-DPAT. Data are mean ± SE for n=6 preparations. There was not a statistically significant difference in area (p=0.637) during the hypercapnic response. A value of 1 implies no change, while values greater than 1 show an increase in frequency, and less than 1 show a relative decrease in frequency.

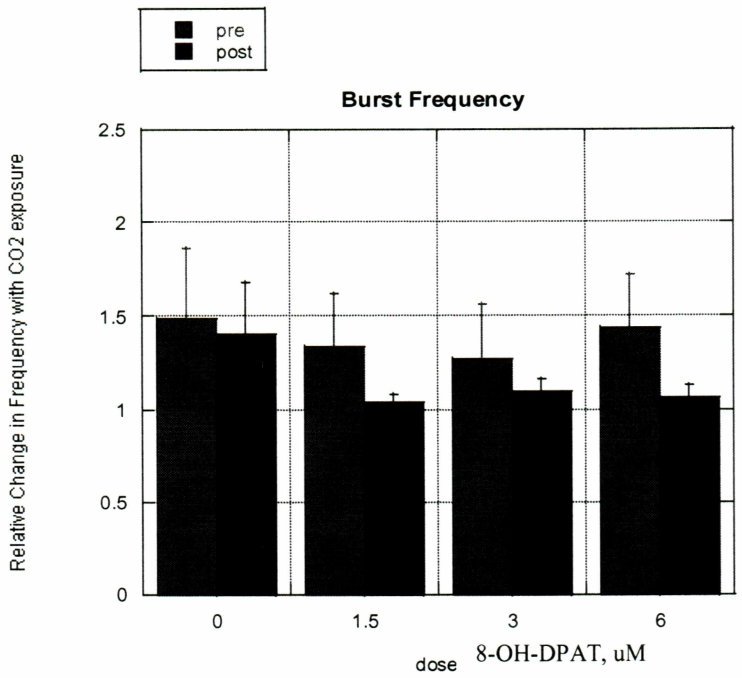

Figure 21. The relative change in burst frequency (Fr) following an increase from 5% to 7% CO_2, before and after treatment with 5HT-1A agonist 8-OH-DPAT. Data are mean ± SE for n=6 preparations. A value of 1 implies no change, while values greater than 1 show an increase in frequency, and less than 1 show a relative decrease in frequency. There was a statistically significant increase in frequency ($p=<0.001$, n=6) during the normal hypercapnic response. The frequency response was significantly reduced ($p=0.018$) following treatment with 8-OH-DPAT, regardless of dose.

4.0 Conclusion

The respiratory centers located in the medulla and pons are responsible for responding to dynamic levels of CO_2 and O_2 (Richerson 2004). The brain must control breathing in a manner that will appropriately compensate for such changes, in order to maintain homeostasis. Nattie and Li (2001) showed that efferent respiratory nuclei in the medulla and pons have no sensitivity to pH on their own; therefore, a mechanism or link must exist that can detect changes in pH and convey this information to the rest of the respiratory network (Feldman *et al.* 2003). One mechanism which may exist is a system of chemoreceptive neurons. Respiratory chemoreceptors transmit information about changing levels of pH in the blood to the rest of the respiratory control network, thus serving to modulate ventilatory responses (Richerson 1995). Several neuron types have been proposed as the long-sought "central chemoreceptors" of the medulla, including neurons that release serotonin as a neurotransmitter (see Guyenet 2004 and Richerson 2005 for review).

This study aimed to define the role of serotonin as a modulator of respiration. Serotonergic neurons possess the necessary requirements to be effective chemoreceptors: they have been shown to be chemosensitive in both *in vitro* and *in vivo* preparations (Wang *et al.* 1998, Wang *et al.* 2001, Richerson 1995, Wang and Richerson 1999, Bradley *et al.* 2002, Putnam *et al.* 2004, Veasey *et al.* 1995, Bernard *et al.* 1996) and they function in areas of the brainstem that are proximal to arteries, where they could readily monitor the acid-base status of blood (Bradley *et al.* 2002; Severson *et al.* 2003; Messier

et al. 2004) and they have anatomical connections that would be expected of a chemoreceptor, including targets in the brainstem that affect respiratory responses. This study tested the hypothesis that brainstem serotonergic neurons are required for respiratory sensitivity to CO_2 in the perfused rat brainstem preparation.

In order to determine the role of serotonergic neurons in chemosensitivity, this study utilized a pharmacological agent (8-OH-DPAT) affecting the serotonergic neurons involved in responding to elevated CO_2. 8-OH-DPAT, a 5-HT_{1A} agonist, selectively binds 5-HT_{1A} receptors which are often autoreceptors located primarily on cell bodies and axon terminals of presynaptic neurons that synthesize and release serotonin (Pauwels 1997). The result of 5-HT_{1A} autoreceptor activation on serotonergic neuron soma is to inhibit neuronal firing. 5-HT_{1A} activation at axon terminals inhibits synaptic release of serotonin (Lalley *et al.* 1994). The net result of 8-OH-DPAT application is to reduce or remove the influence of serotonergic neurons, thereby preventing the activation of post-synaptic neurons that would normally receive serotonergic excitation from these neurons (Lalley 1994).

8-OH-DPAT is not known to significantly affect the respiratory motoneurons under normocapnic conditions (Taylor *et al.* 2005). Results from the present study indicate that although eupnea persists in the absence of serotonergic modulation, the typical chemosensitive response to hypercapnia does not. We abolished the normal increase in frequency associated with CO_2 exposure after removing the influence of serotonergic neurons. These results suggest that serotonergic neurons, although not

essential for the expression of phrenic burst discharge akin to eupnea, are critical for neuroventilatory responses to CO_2.

4.1 Eupnea

The perfused rat brainstem preparation produced phrenic nerve discharge akin to phrenic discharge during normal spontaneous breathing *in vivo* (St. John and Paton 2000). The bursts had a ramp-like, incrementing shape which is consistent with eupneic breathing at normal CO_2 levels (St. John and Paton 2000). Burst responses did not change over time, and repeated hypercapnic challenges did not affect the subsequent normocapnic eupneic breathing pattern. The preparations were consistent among rats. Time controls indicated that these preparations produced strong and steady phrenic burst patterns well beyond the time required to conduct the experiments, ensuring that the protocol could be executed and showing that this experimental preparation provided a robust and appropriate means to model respiration.

4.2 Frequency and Te increase with hypercapnia

The hypercapnic response was consistent with accounts from studies that suggested only a few of the burst variables would change significantly upon exposure to hypercapnic conditions. In initial tests of the perfused rat brainstem preparation, respiratory burst frequency and peak tended to increase with hypercapnia (St. John and Paton 2000). This is consistent with observations of adult rats *in vivo* whose respiratory frequency tends to increase with rising levels of CO_2. When we changed the gas

equilibrating with the perfusate from 5 to 7% CO_2, Fr increased and Te decreased. On the other hand, Ti was unchanged. *In vivo*, many mammalian species tend to breathe at higher frequencies during hypercapnia (St. John and Paton 2000, Nattie 2001); the results from this study indicated that the perfused rat brainstem preparation exhibits a change in ventilatory burst discharge pattern consistent with hypercapnic ventilatory responses of intact mammals (St. John and Paton 2000). In sham treatment experiments, repeated CO_2 challenges produced equally robust neuron-ventilatory responses, indicating that the CO_2 response was unchanged over time, and appeared unchanged despite to prior CO_2 exposures.

4.3 Effect of 8-OH-DPAT on burst pattern during normocapnia.

Under normocapnic conditions, phrenic burst discharge pattern was unaffected following application of 8-OH-DPAT at any dose (1.5, 3, 6uM). In contrast, the addition of 9uM 8-OH-DPAT greatly disrupted baseline eupneic burst pattern such that burst variables and their change with CO_2 exposure could not be adequately assessed. It is possible that introducing a relatively high concentration of agonist had unforeseen side effects and implications beyond the respiratory control system with which this study was concerned.

4.4 Effect of 8-OH-DPAT on respiratory response

Following ANOVA, it was clear that blocking serotonergic transmission with 8-OH-DPAT had no influence on normocapnic burst pattern, but nearly abolished the Fr

response normally seen in untreated animals exposed to hypercapnia. There were no statistically significant differences among different 8-OH-DPAT doses (post hoc Tukey test), and the hypercapnic response was significantly affected by 8-OH-DPAT regardless of what dose was present.

8-OH-DPAT binds $5HT_{1A}$ autoreceptors, preventing serotonergic neuron firing and inhibiting release of serotonin into the synapse. As such, this treatment prevents neurotransmission that would normally activate post-synaptic neurons. These data illustrate that abolishing serotonin signaling results in loss of the frequency response to hypercapnia. Therefore, serotonergic neurons must play a vital role in modulating the respiratory response to CO_2.

4.5 Mechanisms

Past research characterized phrenic discharge patterns in the perfused rat brainstem as identical to eupnea *in vivo* (Paton, 1996, St. John and Paton 2000). This work has established that the perfused rat brainstem preparation preserves the respiratory network responsible for the control of breathing, and therefore, expresses motor patterns resembling those of intact animals. Although higher centers are removed, intact tonic inputs from pulmonary stretch receptors, chemoreceptors, and carotid bodies are able to modify the respiratory response as they would in the intact animal. Thus, use of this preparation provides an opportunity to identify the mechanism of neurotransmission responsible for generation and modulation of breathing.

Past work (St. John and Paton 2000) and data from the present study suggest that the perfused rat brainstem preparation also exhibits changes in burst pattern with hypercapnia that are consistent with hypercapnic ventilatory responses *in vivo*. The present study identifies the critical role of serotonergic neurons in producing neuro-ventilatory responses to hypercapnia and suggests that such mechanisms are also critical for ventilatory responses *in vivo*.

Our experimental results indicate that 8-OH-DPAT treatment did not change normal eupneic burst discharge patterns under normocapnic conditions, but abolished the increase in phrenic burst frequency that normally occurred in response to hypercapnia in this preparation. As such, these results indicate that an 8-OH-DPAT-sensitive mechanism is critical for the neuro-ventilatory response to hypercapnia.

8-OH-DPAT binds to the 5-HT_{1A} autoreceptors on the presynaptic cell. 8-OH-DPAT treatment inhibits serotonergic neuron activity and inhibits release of serotonin by neurons. Both mechanisms prevent serotonergic activation of the post-synaptic cell. As 8-OH-DPAT treatment abolished changes in phrenic burst pattern normally occurring in response to elevated CO_2, our results indicate that serotonergic activation is critical for neuro-ventilatory response to hypercapnia. These data are consistent with serotonergic neurons acting as important chemosensors. Thus, data from this study supports the hypothesis that serotonergic neurons are necessary for the expression of hypercapnic neuro-ventilatory responses in the perfused brainstem preparation and indicate that such neurons could play an important role in CO_2 chemosensitivity *in vivo*.

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